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CONCOMITANT SECRETION OF BIG ENDOTHELIN AND ITS C-TERMINAL FRAGMENT FROM HUMAN AND BOVINE ENDOTHELIAL CELLS

Toshiaki Emori[1], Yukio Hirata[1]*, Kazuki Ohta[1], Masayoshi Shichiri[1], Kentaro Shimokado[2] and Fumiaki Marumo[1]

[1]Second and [2]Third Departments of Internal Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan

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Summary: A specific radioimmunoassay (RIA) for the carboxyl-terminal fragment (CTF) of big porcine endothelin (pET), an intermediate form of pET, was established to characterize big ET-like and its CTF-like immunoreactivity (LI) secreted from cultured bovine and human endothelial cells (EC). The antibody used crossreacted equally with big pET(1-39) and its CTF(22-39), but not with pET(1-21). Serial dilution curves of the culture media from bovine and human EC were parallel to that of standard CTF. Reverse-phase HPLC coupled with RIAs for big ET and ET of the culture media from bovine and human EC revealed essentially the same elution profiles: two major CTF-LI components, one corresponding to big pET(1-39) and the other to its CTF(22-39), in addition to one major ET-LI component corresponding to pET(1-21). The amounts of CTF-LI were almost equal to that of ET-LI on a molar basis. These data suggest that big ET is processed by a putative ET converting enzyme to yield its CTF and the mature ET(1-21) in EC.

A potent vasoconstrictor peptide, termed endothelin (ET), has recently been isolated from the supernatant of cultured porcine aortic endothelial cells (EC) and sequenced (1). Porcine (p) ET consists of 21 amino acid residues with two

* All correspondence should be addressed to Yukio Hirata, M.D., Endocrine Division, Second Department of Internal Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113, Japan.

Abbreviations used in the text: pET, porcine endothelin; hET, human endothelin; big ET, big endothelin; CTF, carboxyl-terminal fragment; RIA, radioimmunoassay; ET-LI, endothelin-like immunoreactivity; ECE, endothelin-converting enzyme; EC, endothelial cells.

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intramolecular disulfide bonds, and it is identical to human (h) ET (2). Sequence analysis of the cDNA coding for pET and hET revealed the presence of the precursor molecules, preproET (1,2). Based on the deduced amino acid sequence, Yanagisawa et al. have postulated that the preproET is initially processed by the endopeptidases specific for the paired dibasic residues to yield an intermediate form, termed big ET. For the production of mature ET(1-21), big pET(1-39) and big hET(1-38) may be processed by the putative ET-converting enzyme (ECE) through an unusual type of proteolytic processing (1).

However, it remains unknown whether big ET is actually synthesized, processed and secreted by EC. To address this question, we have established a radioimmunoassay (RIA) for big ET and characterized the molecular forms of ET-like and big ET-like immunoreactivity (LI) secreted from bovine and human EC in culture.

MATERIALS AND METHODS

Peptides

Big pET(1-39) and its C-terminal fragment(22-39) (CTF) were synthesized by the solid-phase method and purified by ion-exchange chromatography on DEAE-cellulose and reverse-phase HPLC. The homogeneity of the final products was confirmed by analytical reverse-phase HPLC and amino acid analysis. The details will be described elsewhere. pET(1-21) was purchased from Peptide Institute, Inc. (Osaka, Japan).

Cell culture

Bovine EC were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), as previously described (3). Human EC obtained from human umbilical veins (4) were cultured in DMEM containing 20% FCS, 10 ng/ml basic fibroblast growth factor (Amersham International plc, England), and 5 IU/ml heparin. The conditioned medium was collected at 2-3-day intervals, pooled, and stored at -40°C.

RIA for big ET

Synthetic CTF (1 mg) was conjugated to bovine thyroglobulin (10 mg) using carbodiimide as the coupling agent. The conjugate (100 μg) emulsified with complete Freund's adjuvant (Difco Labs., Detroit, MI) was biweekly injected, intradermally, at multiple sites on the back of Japanese white

rabbits. After repeated immunizations, antiserum suitable for RIA was obtained. Synthetic CTF (5 μg) was radioiodinated with [^{125}I]Na (1 mCi; New England Nuclear, Boston, MA) by the lactoperoxidase method, as previously described for the labeling of pET (5). [^{125}I]CTF was purified by adsorption onto a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) and elution with 70% acetonitrile/0.1% trifluoroacetic acid (TFA). The specific activity of [^{125}I]CTF was 100-150 μCi/μg. RIA for big ET was performed essentially in the same manner as reported for ET RIA (3). Briefly, a 0.1 ml sample or standard, 0.1 ml assay buffer, and 0.1 ml anti-CTF-serum (final dilution of 1:60,000) were incubated at 4°C for 24 hrs, followed by the addition of 0.1 ml [^{125}I]CTF (∿10,000 cpm) and further incubation at 4°C for 48 hrs. The bound ligands were separated from the free ligands by the double antibody method.

Extraction and chromatographic procedures

The pooled conditioned media from bovine EC (14-16th passage) and human EC (2-4th passage), acidified with TFA were applied to a Sep-Pak C_{18} cartridge which had been prewashed sequentially with methanol, distilled water, and 0.1% TFA. The materials adsorbed to the cartridge were eluted with 70% acetonitrile/0.1% TFA. The recoveries of standard pET and big pET during the extraction procedure were 77% and 59%, respectively. An aliquot of the extracts was subjected to reverse-phase HPLC, using an octadecyl-silica column (0.45x25 cm, JASCO, Tokyo, Japan), which was eluted with a linear gradient (15-60%) of acetonitrile in 0.09% TFA for 60 min with a flow rate of 1 ml/min. One-ml fractions were collected and subjected to RIAs for big ET and ET (3). The recoveries of standard pET, big pET, and its CTF during reverse-phase HPLC were 96%, 89%, and 90%, respectively.

RESULTS

As shown in Fig. 1, the minimum detectable quantity of big ET RIA was 10 fmol/tube, and the 50% intercept was 70 fmol/tube. The antibody used in the present RIA crossreacted equally with big pET(1-39) and pCTF(22-39) on a molar basis, but not with pET(1-21) (Fig. 1); it had no crossreactivities with angiotensin II, arginine-vasopressin, or atrial natriuretic peptide. Serial dilution curves of the conditioned media from both bovine and human EC were parallel to that of standard CTF (Fig. 2).

As shown in Fig. 3, the reverse-phase HPLC elution profiles of ET-LI and CTF-LI in extracts of the conditioned media from both bovine and human EC by were essentially similar. A single major component with ET-LI was eluted at a

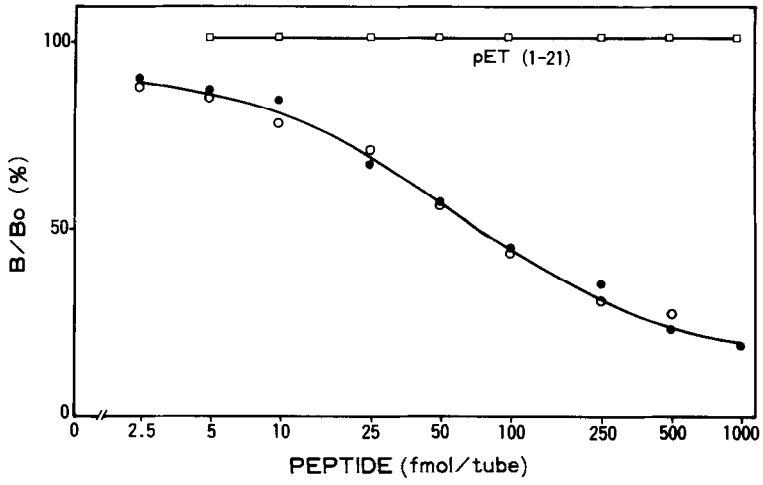

Fig. 1. Crossreactivities of ET and related peptides in big ET RIA.
Serial dilutions of big pET (●), pCTF (○) and pET (□) in big ET RIA are shown. The total binding was 18% of the added [^{125}I]CTF; the nonspecific binding was 3.8±0.9% of the total binding (n=7).

position corresponding to that of standard pET(1-21). In contrast, two major components with CTF-LI were observed: one component eluted slightly earlier than pET, which corresponded to the elution position of standard big pET(1-39), and the other component eluted much earlier than pET, which corresponded to the elution position of standard pCTF(22-39).

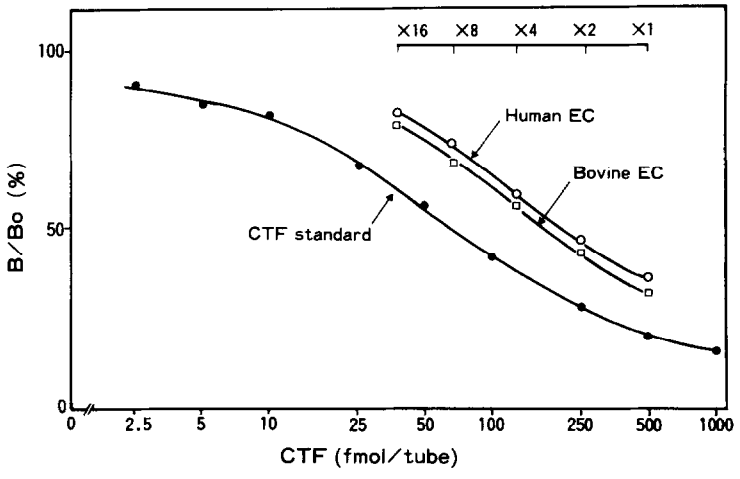

Fig. 2. Serial dilution curves of the conditioned media from bovine and human EC in big ET RIA.
Dilution curves of the conditioned media from bovine (□) and human (○) EC are compared to that of standard CTF of big pET (●).

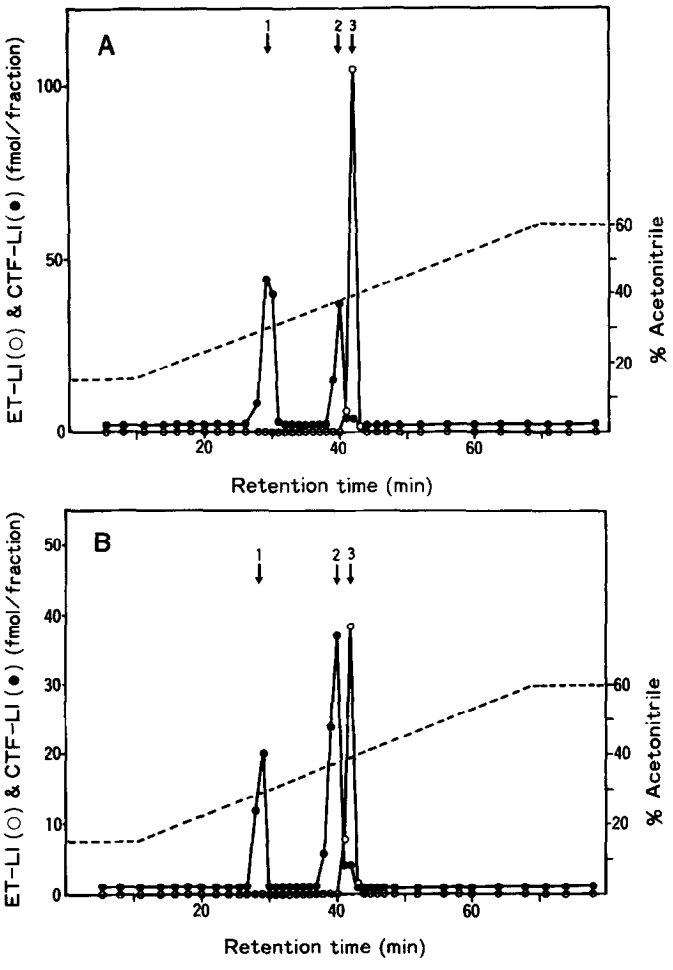

Fig. 3. Reverse-phase HPLC profiles of the conditioned media from cultured bovine and human EC.
Elution profiles of ET-LI (○) and CTF-LI (●) in the cultured media from bovine EC (A) and human EC (B) are shown. Arrows indicate the elution positions of standard pCTF(22-39) (1), big pET(1-39) (2), and pET(1-21) (3), respectively. Dotted lines denote the linear gradient(15-60%) of acetonitrile.

While the total amount of ET-LI in the culture media from both bovine and human EC was almost equal to that of CTF-LI on a molar basis, the amount of big ET-LI in the medium from human EC was greater than that of bovine EC.

DISCUSSION

In the present study, we have successfully established a specific RIA for big ET. The antibody generated against synthetic pCTF(22-39) has full crossreactivity with big pET(1-

39), but not with pET(1-21). From the present results, serial dilutions of the conditioned media from both bovine and human EC in culture appear to be parallel to that of standard CTF, suggesting that big ET-LI and/or its CTF-LI are/is also secreted from bovine and human EC.

Reverse-phase HPLC coupled with RIAs for ET and big ET revealed essentially the similar elution profiles of ET-LI and CTF-LI in the extracts of media from bovine and human EC: one single peak with ET-LI corresponding to standard pET(1-21) and two peaks with CTF-LI corresponding to standard big pET(1-39) and its CTF(22-39), respectively.

Yanagisawa *et al.* (1) have postulated that the 203-residue prepropET, containing two paired basic amino acid residues (Lys^{51}-Arg^{52} and Arg^{92}-Arg^{93}), may be initially converted to big pET(1-39) by the processing endopeptidases, which is then cleaved to the mature pET(1-21) by the putative ECE through an unusual proteolytic processing between Trp^{21} and Val^{22}. The present results demonstrating almost equimolar amounts of ET(1-21)-LI and CTF(22-39)-LI in the cultured media from both bovine and human EC, lend strong support to their hypothesis (1) that big pET(1-39) is an intermediate form that is cleaved into its N-terminal fragment, *i.e.*, pET(1-21), and CTF(22-39) by the ECE. However, the amount of big ET-LI in the medium from human EC is greater than that from bovine EC. This may be accounted for either by the different ECE activities between bovine and human EC or the different passages of cultured EC used in the experiments.

The concomitant secretion of big ET-LI and its CTF-LI from cultured EC *in vitro* also suggests that big ET and its CTF may be secreted along with ET into the circulation. These data are consistent with our recent observations that the

major circulating ET-LI in human plasma consists of two forms, one corresponding to ET(1-21) and the other to big ET (6). It is thus anticipated that CTF-LI of big ET may also circulate in parallel with ET-LI, in analogy to the cosecretion of proinsulin C-peptide and insulin (7).

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